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Research Article

Rapid and Reliable HPLC Method for the Determination of Vitamin C in Pharmaceutical Samples

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Abstract

Purpose: *To develop and validate an accurate, sensitive and reproducible high performance liquid chromatographic (HPLC) method for the quantitation of vitamin C in pharmaceutical samples.*
Method: *The drug and the standard were eluted from Superspher RP-18 (250 mm x 4.6 mm, 10μm particle size) at 20 ^{0}C. The mobile phase was prepared by carefully adding acetic acid (500 ml) to 1.5g of 1-hexanesulfonic acid sodium salt and mixing well (pH 2.6). The flow rate was 0.7 mL min^{-1}. UV detector, set at 280 nm, was used to monitor the effluent.*
Results: *Each analysis required no longer than 4 min. The limit of quantitation was 1.95 μg mL^{-1}. Recovery (%) for different concentrations ranged from 99.58 to 101.93.*
Conclusion*: The simplicity of this low-cost, rapid technique and its high specificity to ascorbic acid, even in the presence of a variety of excipients, demonstrate that this HPLC method would be particularly suitable for the determination of ascorbic acid in the investigated preparations as well as other similar pharmaceutical/veterinary formulations without prior sample preparation.*

Keywords*: Ascorbic acid; Pharmaceutical preparations; HPLC method*

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INTRODUCTION

Vitamin C (ascorbic acid, ascorbate, AA) is a water-soluble organic compound involved in many biological processes [1]. Although all the functions of AA have not been fully elucidated, it is likely that it is also involved in maintaining the reduced state of metal cofactors, for example, monooxygenase (Cu^{+}) and dioxygenase (Fe^{2+}). In cells, the other role of AA is to reduce hydrogen peroxide (H_2O_2), which preserves cells against reactive oxygen species [2]. Primates and several other mammals are not able to synthesis ascorbic acid. The only way humans can obtain ascorbic acid is via food, but the exact daily requirements of vitamin C for humans are not yet clear. Currently, the estimated average requirement and recommended dietary allowance of ascorbic acid are 100 and 120 mg per day, respectively [3].

Many analytical techniques including sensors and biosensors [4-6] have been suggested for the detection of ascorbic acid in various types of samples. Integrated methods, utilizing flow injection analysis, high performance liquid chromatography [7-9] or capillary electrophoresis [10-13] and a detector, are mostly employed for the determination of vitamin C. However, some of these methods are time-consuming, while others are costly, require special training for operators of the equipment, or suffer from insufficient sensitivity or selectivity.

Vitamin C has been widely employed in pharmaceutical and cosmetic preparations to protect them against oxidation and to exert physiological/biological activities. In view of the fact that pharmaceutical dosage forms usually contain a variety of excipients that may appear as interferents, as well as the likelihood of the presence of degradation products and/or stabilizing antioxidant agents for vitamin C, HPLC method possesses advantages. HPLC is considered a sensitive and selective method and therefore suitable for active substance determination; it is also suitable for the evaluation of stability in formulations in the pharmaceutical and cosmetic industries [14].

The purpose of this study, therefore, was to develop and validate a HPLC method for the quantitation of vitamin C (ascorbic acid) in pharmaceutical powder or tablet preparations containing various excipients, without prior sample preparation.

EXPERIMENTAL

HPLC apparatus and conditions

The HPLC system used consisted of a Hewlet Packard HPLC 1100 Series isocratic LC system with diode array detector (DAD) and flame-photometric detector (FLD). Prior to performing the validation assay, chromatographic conditions for the HPLC method were studied in order to achieve appropriate system suitability. Mobile phase composition was tested with methanol /phosphate buffer + tetrabutylammonium (30:70 and 70:30, v/v) in C8 column (λ = 254 nm); and 0.2 % metaphosphoric acid in water solution, 0.2% metaphosphoric acid/methanol (95:5 and 90:10, v/v), 0.2% metaphosphoric acid /acetonitrile (95:5 and 90:10, v/v) and 0.2% metaphosphoric acid/methanol /acetonitrile (90:5:5, v/v/v) in C18 column (λ = 254 nm).

The column used was Superspher RP-18 (250 x 4.6mm) while the mobile phase (pH 2.6) consisted of 1.5 g dissolved in 500 ml of acetic acid (99,8%) and mixed well. Routine degassing of the mobile phase was carried out by passing it through a 0.45 µm membrane filter (Millipore, Bedford, MA, USA). The mobile phase was pumped isocratically at a flow rate of 0.7 mL min^{-1} at 20 ^{0}C. This low operating temperature was used because the stability of ascorbic acid decreases with increasing temperature The injection volume was 50 µL.

Reagents and chemicals

Ascorbic acid ($C_6H_8O_6$) with 99.0% purity was kindly provided by F. Hoffmann–La Roche Ltd. Basel, Swiss.. Doxycycline was obtained from PromoChem, Teddington, United Kingdom. All the solvents used were of HPLC grade while the other chemicals were of spectroscopic grade and obtained from Merck (Darmstadt, Germany). Pure water was produced with a Millipore Milli-Q Plus System (Molsheim, France). All the reagents were used without any further purification. Branded pharmaceutical and veterinary formulations, in powder or tablet form, were obtained from commercial sources and used as received, without any further purification. The composition of the preparations are as follows:

Vetadox powder: Supplied by "Actavis" Leskovac, Serbia, it consists of various ingredients, among them doxycycline hyclate (the only active component; content, 250 mg), vitamin C (50 mg) as anti-oxidant and glucose as sweetner.

Ferveks for adults (Bristol-Myer Squibb-New York, USA in powder form and contained pheniramin (25 mg), paracetamol (500 mg), vitamin C (200 mg) and flavor (25mg)

Eferalgan (Bristol-Myer Squibb, New York, USA), an effervescent tablet, contained paracetamol (330 mg), vitamin C (200 mg), and $KHCO_3$, $NaHCO_3$, sorbitol, citric acid anhydride, sodium-benzoate, sodium docusate and povidone as excipients.

Ca+C-vitamin (Innopharm, Budapest, Hungary), an effervescent tablet, contained Ca (300 mg, 37.5 %) in the form of calcium carbonate, vitamin C (60 mg), as well as $CaCO_3$, $NaHCO_3$, sorbitol, citric acid anhydride, Na-cyclamate, Na-saccharin , synthetic beta carotene and flavor as excipients

Linearity assay

Approximately 50 mg of standard ascorbic acid (99 % purity) was weighed precisely and dissolved in 50 mL of water-acetic acid clear mixture (20:1, v/v) to obtain a stock concentration of 1 $mgmL^{-1}$. Standards were freshly prepared. To obtain the working solution, aliquots of standard ascorbic acid solution were diluted to a concentration of 0.2 mg ml^{-1}. The working standard solutions were prepared in duplicate, filtered and degassed by passing them through a 0.45 μm membrane filter (Millipore, Bedford, MA, USA). The linearity study verifies that the sample solutions are in a concentration range where analyte response is linearly proportional to the concentration. To establish linearity of the proposed methods, five separate series of ascorbic acid solutions were prepared from the stock solutions and analyzed. Least square regression analysis was done for the data obtained. The linearity was studied over a concentration range of 0.15 – 0.25 mg mL^{-1}. Replicates of three injections were performed for each sample. Linearity data were computed on a personal computer using Microsoft Excel program (version 2003, Microsoft Co., Redmond, USA).

Accuracy/recovery and precision assay

The accuracy of the method is the closeness of the measured value to the true value for the sample. Accuracy was assessed as percent relative error and mean % recovery.

Approximately 40, 50 and 60 mg of standard ascorbic acid were weighed precisely and dissolved, separately, in 50mL of the mobile phase. To achieve accuracy/recovery, aliquots of these samples were diluted to appropriate final concentrations of ascorbic acid solution [15], i.e., 2 ml of each solution diluted to 10 ml with mobile phase solution. The accuracy of the method was checked by determining recovery values. Accuracy/ recovery was calculated for six runs of each solution.

The precision was determined by measuring five sample probes under the same experimental conditions. To calculate precision, intra- and inter-day tests were performed and the results were expressed as relative standard deviation (RSD, %).

Limits of detection (LOD) and quantitation (LOQ) assay

The limits of detection and quantitation were determined by serial dilutions of ascorbic acid solutions in order to obtain signal/noise ratios of ≈ 3:1 for LOD and ≈ 10:1 for LOQ.

Approximately 25 mg of standard ascorbic acid was weighed precisely and dissolved in 50 mL of the mobile phase. Appropriate amounts of standard ascorbic acid solution were diluted to the required concentrations of 0.5, 1.0, 1.5 and 2 µg mL^{-1}. Working standard solutions were prepared in triplicate.

Selectivity assay

The specificity of the HPLC method for ascorbic acid quantitation in pharmaceutical/ veterinary preparations was investigated in order to obtain an indication of possible interference from excipients in topical preparations.

For specificity and selectivity of method, ascorbic acid solutions (0.2 µg ml^{-1}) were prepared in the mobile phase along with and without common ingredients (doxycycline hyclate, pheniramin, paracetamol, $CaCO_3$, $KHCO_3$, $NaHCO_3$, sorbitol, citric acid anhydride, sodium-benzoate, sodium docusate, povidone, Na-cyclamate, Na-saccharin, synthetic beta carotene) separately. All the solutions were injected into the Superspher RP-18 (250 x 4.6mm) column. In this assay, it was tested by running solutions containing placebo (using the same quantities and conditions as for the test samples) to verify that there is no overlapping peak at the retention times corresponding to those of the analytes. Paired *t*-test at 95 % level of significance was performed to compare the area of the peaks.

Sample preparation

Approximately 250 mg of each topical formulation was weighed precisely and dissolved separately in 50 mL of the mobile phase. The mixtures were centrifuged at 3000 rpm for 5 min at room temperature (20 ^0C). The supernatants were collected and aliquots of the samples were diluted to appropriate final concentration (0,2mg ml^{-1}).

RESULTS

Mobile phase

The mobile phase (pH 2.6) prepared by carefully adding acetic acid (500 mL) to 1.5 g of 1-hexanesulfonic acid sodium salt thorough mixing gave good response and a retention time of 4 min for ascorbic acid. The other mobile phases tested did not present adequate response for ascorbic acid quantitation since they were not able to identify the antibiotic, doxycycline, as a component of the pharmaceutical preparations, and also provided unsuitable retention time for the active substance (≈ 1 min) [16].

Linearity

The linearity was checked on samples of standard ascorbic acid at five different concentrations (0.25 – 1.5 mg mL^{-1}). The regression equation derived was: y = 10245x – 89.95 with a correlation coefficient (R^2) of 0.9998, where *x* represents concentration in µg $ml–^1$, and *y* represents the HPLC peak area, which was automatically measured by an integrator of the HPLC instrument. Linearity data were computed on a personal computer using Microsoft Excel program (version 2003, Microsoft Corp., Redmond, USA).

Accuracy/recovery and precision

Accuracy/recovery was calculated for three runs of each solution. The results of accuracy/recovery and precision experiments

are recorded in Table 1. The data indicate an adequate percentage of accuracy/recovery for the HPLC method for the quantitation of ascorbic acid in the pharmaceutical preparations [17,18].

Table 1: Accuracy of the HPLC method for ascorbic acid determination (N = 3)

Concen-tration (mg/g)	Recover y (%) X	X ±SD	RSD (%)
40	102.43	102.39±0.049	0.048
	102.42		
	102.34		
50	102.03	101.70±0.450	0.442
	101.89		
	101.19		
60	100.09	100.07±0.020	0.020
	100.05		
	100.07		

Limits of detection (LOD) and quantitation (LOQ)

LOD, which is defined as the lowest active substance concentration that can be determined by a method, usually cannot be calculated precisely and accurately. On the other hand, LOQ is the concentration of the sample used in analysis that can be obtained with adequate precision and accuracy. An estimation of the limits, which was achieved by the determination of the signal/noise ratios of 3:1 (LOD) and 10:1 (LOQ), were ≈ 1.95 and ≈ 6.5 μgmL^{-1}, for LOD and LOQ, respectively.

Specificity

The specificity of the HPLC method for vitamin C quantitation in the pharmaceutical formulations is an indication of possible interference from excipients in the preparations. The presence of other ingredients, including the antibiotics, in the formulations did not cause any interference with the ascorbic acid peak. Under the test conditions, ascorbic acid was observed to be well resolved from the other components of the formulations and potential degradation products of vitamin C. Thus the method is specific for vitamin C.

Application of the developed method to vitamin C formulations

The outcome of the application of the developed method to the determination of vitamin C in some pharmaceutical formulations are shown in Figures 1 and 2. The representative chromatograms of the standard sample of vitamin C and the test preparations show identical retention times.

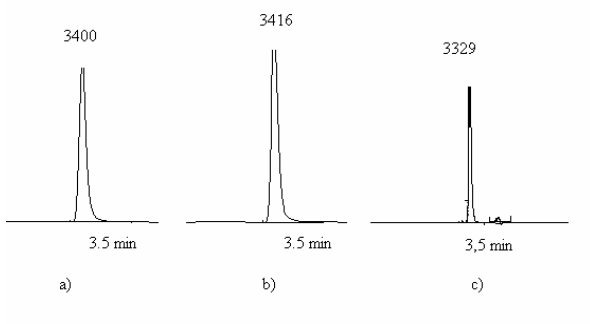

Fig 1: Representative chromatograms of (a) standard vitamin C, (b) Vetadox powder and (c) Ferveks powder

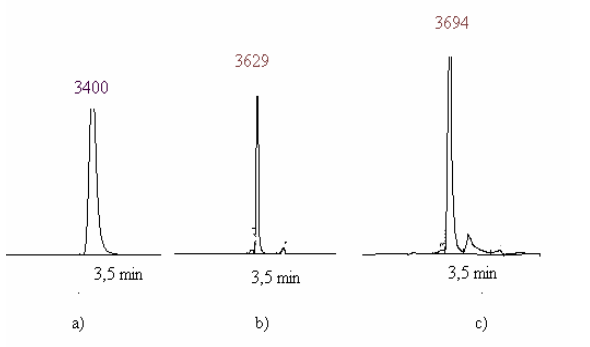

Fig 2: Representative chromatograms of (a) standard vitamin C, (b) Eferalgan powder, (c) Ca-C-vitamin powder

Assay results for the determination of ascorbic acid in commercial pharmaceutical are shown in Table 3. RSD (%) indicates the accuracy of determination of ascorbic acid in the investigated pharmaceutical preparations

Table 3: Assay results for the determination of ascorbic acid in some commercial pharmaceutical preparations

Preparation	Labelled content (mg)	Actual content ±SD (mg)	RSD (%)
Vetadox	50	49.52 ± 0.93	1.87
Ferveks	200	190.05 ± 3.32	1.36
Eferalgan	200	192.78 ± 1.46	1.46
Ca-C-vitamin	200	207.73 ± 1.02	0.42

DISSCUSION

The mobile phase used increased retention time to ≈3.5 min, providing a satisfactory resolution of ascorbic acid from doxycycline and the excipients present in the formulations, with sharp and symmetrical peaks, and also minimal interferences from equipment noise. In addition, the mobile phase maintained long column life.

The results demonstrated satisfactory and consistent performance of the HPLC method. Least-squares regression analysis used to evaluate the concentration range data indicate excellent linearity over the interval studied (0.25 - 1.5 mg mL^{-1}), The R^2 obtained for this ascorbic acid concentration range was ≥ 0.99.

The low values of RSD (0.38 and 1.22 %, respectively) are evidence of the good precision of the developed HPLC method and also indicates non-variability of the data. LOD and LOQ values of ≈1.95 and ≈6.5 µg mL^{-1}, respectively, indicate that the method is sensitive.

The presence of the other ingredients in the formulations, including an antibiotic (doxycycline) did not cause any interference with ascorbic acid peak. Ascorbic acid was well resolved from the other components of the formulations and the potential degradation product of vitamin C. Thus, the method is selective for vitamin C.

The relative standard deviation (RSD) of 1.87 % was low, being less than < RSD_{max}. Thus the method is precise. Overall, the results show that the proposed method can be successfully applied for the determination of vitamin C in pharmaceutical preparations.

CONCLUSION

The developed HPLC method for the determination of ascorbic acid in pharmaceutical preparations containing various other ingredients including excipients (doxycycline hydrochloride, pheniramin, paracetamol, etc) has been validated for linearity, sufficient accuracy/recovery and precision, as well as low values of limits of detection and quantitation. The method provides a rapid, sensitive, accurate, and reproducible means of determining vitamin C in pharmaceutical and veterinary formulations without prior sample preparation.

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REFERENCES

1. *Velisek J, Cejpek K. Biosynthesis of food constituents: Vitamins. 2. Water-soluble vitamins: part 1 - a review. Czech. J. Food Sci. 2007; 25: 49-64.*
2. *Linster CL, Van Schaftingen E. Vitamin C - Biosynthesis, recycling and degradation in Mammals, Febs J., 2007; 274: 1-22.*
3. *Englard S, Seifter S. The Biochemical Functions of Ascorbic-Acid. Annu. Rev. Nutr. 1986; 6:365-406.*
4. *Cofan, C, Radovan C. Simultaneous chronoamperometric sensing of ascorbic acid and*

acetaminophen at a boron-doped diamond electrode. Sensors 2008; 8: 3952-3969.

5. *Wang Y, Xu H, Zhang JM, Li G. Electrochemical sensors for clinic analysis. Sensors, 2008; 8: 2043-2081.*
6. *Yogeswaran U, Chen SM. A review on the electrochemical sensors and biosensors composed of nanowires as sensing material. Sensors 2008; 8:290-313.*
7. *Behrens WA, Madere RA. Highly Sensitive High-Performance Liquid-Chromatography Method for the Estimation of Ascorbic and Dehydroascorbic Acid in Tissues, Biological-Fluids and Foods. Anal. Biochem. 1987; 165: 102-107.*
8. *Shakya R, Navarre DA. Rapid screening of ascorbic acid, glycoalkaloids, and phenolics in potato using high-performance liquid chromatography. J. Agric. Food Chem. 2006; 54: 5253-5260.*
9. *Melendez-Martinez AJ, Vicario IM, Heredia F. J. Provitamin A carotenoids and ascorbic acid contents of the different types of orange juices marketed in Spain. Food Chem., 2007, 101: 177-184.*
10. *Wang J, Chatrathi MP, Tian BM, Polsky R. Microfabricated electrophoresis chips for simultaneous bioassays of glucose, uric acid, ascorbic acid, and acetaminophen. Anal. Chem., 2000; 72: 2514-2518.*
11. *Davey MW, Bauw G, VanMontagu M. Simultaneous high-performance capillary*

electrophoresis analysis of the reduced and oxidised forms of ascorbate and glutathione. J. Chromatogr. B, 1997; 697: 269-276.

12. *Klejdus B, Petrlova J, Potesil D, Adam V, Mikelova R, Vacek J, Kizek R, Kuban V. Simultaneous determination of water- and fat-soluble vitamins in pharmaceutical preparations by high-performance liquid chromatography coupled with diode array detection., Anal. Chim. Acta, 2004; 520: 57-67.*
13. *Wu T, Guan YQ, Ye JN. Determination of flavonoids and ascorbic acid in grapefruit peel and juice by capillary electrophoresis with electrochemical detection. Food Chem. 2007; 100: 1573-1579.*
14. *Marshall PA, Trenerry VC, Thompson CO.* The determination of total ascorbic acid in beers, wines, and fruit drinks by micellar electrokinetic capillary chromatography *J. Chromatogr. Sci., 1995; 33: 426-431*
15. *Guide for Validation of Analytical and Bioanalytical Methods, Resolution R.E. n. 899. Brazilian Sanitary Surveillance Agency, Bras´ılia, DF, 2003.*
16. *Wagner ES, Barry L, Coffin RD. High-performance liquid chromatographic determination of ascorbic acid in urine: effect on urinary excretion profiles after oral and intravenous administration of vitamin C. J Chromatogr. 1979; 163(2): 225-229.*
17. *Polesello S. How to present an analytical method* , *Food Chem, 1997; 58: 145-147*
18. *Analytical Procedures and Method Validation. US Food and Drug Administration,* Rockville, MD, 2000.